DIFFERENTIAL EXPRESSION OF GENES IN CLINICAL STRAINS OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPONSE TO ISONIAZID

by
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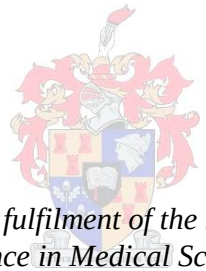

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Medical Sciences (Medical Biochemisty) at the University of Stellenbosch

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Isoniazid forms part of the first line anti-tuberculosis therapy and it is generally used to treat latent *Mycobacterium tuberculosis* infection. Isoniazid inhibits synthesis of long chain mycolic acids found in cell wall of *Mycobacterium tuberculosis*, which have proven vital for the survival of the bacterium. Mycolic acids are primarily synthesized by the fatty acid synthase enzyme (FAS) system found in mycobacteria as the FAS-I and FAS-II complex. Isoniazid kills the bacteria by blocking the FAS-II complex, required for extension of mycolates. It does this by entering the tubercle bacilli as a prodrug where isoniazid becomes activated by catalase peroxidase encoded by *katG* gene. The activated isoniazid then forms a complex with NAD^+ which targets InhA (NADH-dependent enoyl-acyl carrier protein reductase) located in the FAS-II complex. Loss of catalase peroxidase, due to gene mutations or a complete *katG* gene deletion is one of the primary mechanisms conferring resistance to INH in *Mycobacterium tuberculosis*. In addition, four other genes (*inhA*, *KasA*, *ndh* and *ahpC*) are also associated with INH resistance. Nonetheless, mutations in these five genes are present in only 70-80% of INH resistant clinical isolates, implying that other mechanisms are involved in resistance of *Mycobacterium tuberculosis* to isoniazid.

This study aims to quantify the expression level of genes induced by isoniazid in the mycolic acid pathway and drug transport in two closely related *Mycobacterium tuberculosis* Beijing cluster 208 isolates. These are the fully susceptible (K636) and isoniazid mono-resistance strains (R55), with minimum inhibitory concentrations of 0.1 and 4 µg/ml, respectively. Both these isolate had no isoniazid gene associated mutations. The isolates were cultured in the presence and absence of 0.1µg/ml isoniazid for 24 hours after which RNA was extracted followed by QRT-PCR analysis to identify differentially expressed genes.

This result has shown that various genes were differentially expressed in response to low level INH exposure. The most significant up-regulation was observed in genes (*acpM*, *fabD*, *Accd6* and *fbpC*) encoding the FAS-II complex and genes (*efpA*, *iniA*, *iniB*, and *mmpl7*) involved in drug transport. In addition, two genes (*ndh* and *fbpC*) were significantly down-regulated in the isoniazid mono-resistant isolate. Based on these findings, we propose a model whereby isoniazid exposure in the susceptible isolate inhibits FAS-II complex and with its associated accumulation in mycolates kills the bacterium. In contrast, we propose that in the resistance isolate the bacterium acquires additional resistance by the activation of efflux pumps in combination with disruption in INH-NAD^+ complex formation that protect inhibition of InhA located in FAS-II complex.

OPSOMMING

Isoniasied vorm deel van die eerste linie van behandeling teen tuberkulose en word algemeen gebruik om latente *Mycobacterium tuberculosis* infeksie te behandel. Isoniasied inhibeer die sintese van langketting mikolitiese sure wat in die selwand van *Mycobacterium tuberculosis* voorkom. Dit is bewys dat hierdie sure essensiëel is vir die oorlewing van die bakterie. Mikolitiese sure word hoofsaaklik gesintetiseer deur die vetsuur sintase ensiem (FAS) sisteem wat in mikobakterieë voorkom as die FAS-I en FAS-II komplekse. Isoniasied dood die bakterieë deur die FAS-II kompleks, wat nodig is om die verlenging van mikoliete, te blokkeer. Dit word bewerkstellig deurdat ‘n pro-vorm van die middel die tuberkulose bacilli binnedring, waarna isoniasied geaktiveer word deur katalase peroksidase, wat deur die *katG* geen geënkodeer word. Die geaktiveerde isoniasied vorm ‘n kompleks met NAD^+, wat InhA (NADH-afhanklike enoïasiel draer proteïenreduktase), geleë in die FAS-II kompleks teiken. Een van die primêre meganismes wat weerstandigheid teen isoniasied bewerkstellig, is die verlies van katalase peroksidase weens geenmutasies of algehele delesie van die *katG* geen. ‘n verdere vier gene (*inhA, kasA, ndh* en *ahpC*) word ook verbind met isoniasied weerstandigheid. Nietemin is mutasies in hierdie vyf gene teenwoordig in slegs 70-80% van isoniasied weerstandige kliniese isolate, wat impliseer dat ander meganismes ook betrokke is in die weerstandigheid van *Mycobacterium tuberculosis* teen isoniasied.

Die doel van hierdie studie is om die vlak van uitdrukking van gene wat deur isoniasied in die mikolitiese suur biochemiese pad geïnduseer word, asook middel transport te kwantifiseer in twee naby verwante *Mycobacterium tuberculosis* isolate van Beijing groep 208. Die twee isolate is die volledig sensitiewe (K636) en isoniasied monoweerstandige (R55), met minimum inhiberende konsentrasies van onderskeidelik 0.1 en 4µg/ml. Mutasies wat geassosiëer word met isoniasied weerstandigheid was afwesig in beide die isolate. Kulture is

van die isolate gemaak met en sonder 0.1µg/ml isoniasied vir 24 uur, waarna RNA geëkstraeer is deur middel van QRT-PCR analise om gene te identifiseer wat verskillend uitgedruk word.

Die resultate toon dat verskeie gene verskillend uitgedruk is in reaksie op laevlak isoniasied blootstelling. Die mees prominente opregulering is waargeneem in die gene (*acpM, fabD, accd6* en *fbpC*) wat die FAS-II kompleks enkodeer, asook die gene (*efpA, iniA, iniB* en *mmpl7*) wat betrokke is in middel transport. Beduidende afregulering van ‘n verdere twee gene in die isoniasied monoweerstandige isolate, naamlik *ndh* en *fbpC* is waargeneem. Op grond van hierdie waarnemings, stel ons ‘n model voor waarvolgens isoniasied blootstelling in die sensitiewe isolaat die FAS-II kompleks inhibeer, en met die gevolglike akkumulasie van mikoliete, dood dit die bakterium. In teenstelling stel ons voor dat addisionele weerstandigheid bekom word in die weerstandige isolaat deur die aktivering van uitvloeipompe, in kombinasie met die ontwrigting van die INH-NAD^+ kompleksvorming wat die inhibisie van InhA binne die FAS-II kompleks beskerm.

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  My heart leaps for joy and I will give thanks to Him in song. ~Psalm 28:7

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Chapter 1

1.1 BACKGROUND

Tuberculosis (TB), caused by an acid-fast bacillus *Mycobacterium tuberculosis* (*M.tuberculosis*) (18) leads to 1.7 million deaths annually (24,26). The global TB incidence is further aggravated by emergence of human immunodeficiency virus (HIV), which accounts for 15% of global TB burden (24). Although, the rate of TB incidence is slowly declining at less than 1% per year (26), African countries are still experiencing high burden of TB, fuelled by the high prevalence of HIV and an increase in population (24-26). Current evidence-based short course regimens of chemotherapy for TB have been effective in curing TB, especially under the management of Direct Observed Therapy, short course (DOTS) and its successor the STOP TB strategy (8,19,21). The main purpose of this strategy is to treat infected patients, prevent transmission and development of drug resistance TB (7). DOTS was implemented by the World health Organization (WHO) in the mid-1990's and was adopted in South Africa in 1996 (17,26). Since its implementation, treatment of smear positive cases reached the global target of 85% for the first time in 2007 (8,24-26).

Streptomycin (STR) was the first effective anti-TB drug used for the treatment of TB. However soon after its introduction in 1944, resistance to the drug was observed in clinical isolates of *M.tuberculosis* (4,11). Resistance to anti-TB drugs in *M.tuberculosis* is due to spontaneous chromosomal mutations as genetic elements such as plasmids and transposons do not mediate drug resistance in pathogen (5,18,27). Subsequent spread of these resistant

M.tuberculosis strains from the index patients to others, exacerbate the resistant TB epidemic (23).

More recently whole genome sequencing of various *M.tuberculosis* strains, including drug resistance strains, have been completed to further investigate the evolution of drug resistance (2,12). To date, a total of 14 genes were found to be associated with resistance to anti-TB drugs: isoniazid (INH) [*katG, inhA, ndh, ahpC,* and *KasA*], rifampicin (RIF) [*rpoB*], pyrazinamide (PZA) [*pncA*], ethambutol (EMB) [*embA, embB and embC*], STR, capreomycin (CAP), kanamycin (KAN), amikacin (AMI) [*rpsL* and *rrs*] and fluoroquinolones (FQs) [*gyrA* and *gyrB*] (18,22). Recently a database was established listing mutations associated with *M.tuberculosis* drug resistance and frequencies of the most common mutation associated with resistance to specific drugs (20).

M.tuberculosis strains, resistant to at least INH and RIF are defined as multi-drug resistant TB (MDR-TB) (3). Data collected over the past decade from over 100 countries indicate that 5% of all TB cases have MDR-TB (26). The highest rate of MDR-TB recorded was 500 000 in 2008, where 1 in 10 of MDR-TB was classified as extensively drug resistant (XDR-TB) (24,26). XDR-TB is defined as an MDR isolate with additional resistance to one of the FQs and any one of the three injectable second line anti-TB drugs (KAN, AMI and CAP) (10,15). The emergence of MDR-TB and XDR-TB place additional pressure on TB control programs and introduce fears of TB epidemic turning into a drug resistance epidemic (6).

INH was discovered in 1952, and still remains one of the forefront chemotherapeutic agents to fight TB (14). This is mainly due to its high activity against dividing *M.tuberculosis* (28). INH primarily targets the mycolic acid pathway and ultimately results in inhibition of biosynthesis of mycolic acids (13). Resistance of *M.tuberculosis* to INH is as results of various genomic mutations found in five genes (*katG*, *inhA*, *kasA*, *ndh* and *ahpC*) (18). Most

prominent of these mutations is the S315T found in *katG* gene which is the major mechanism of INH resistance in *M.tuberculosis* and mutation in *inhA* gene or its promoter region found at position -15(C-T) (9,16). However these resistance mutations have been found to be absent in approximately 20-30% of INH resistance clinical isolates (18). INH mono-resistance or resistance in combination with other drugs is now the second most common type of resistance globally, accounting for 13% of all new and previously treated TB cases (1,26).

1.2 PROBLEM STATEMENT:

The mechanism of action of INH is still poorly defined and it is therefore important to further comprehend the protective mechanisms of *M.tuberculosis* against INH.

1.3 OVERALL HYPOTHESIS:

In this study we hypothesize that *M.tuberculosis* protects itself against INH by an interplay between intrinsic and extrinsic mechanism and also by detoxification of INH.

1.4 AIMS OF THE STUDY:

1. To quantify the expression level of genes induced by INH in the mycolic acid pathway in an INH susceptible and INH mono resistant *M.tuberculosis* Beijing strain genotype without any INH gene associated mutations.
2. To investigate efflux pumps as an additional protective mechanism against INH.

1.5 EXPERIMENTAL APPROACH:

Genotypically closely related Beijing cluster 208, INH susceptible (K636) and INH-mono resistant (R55) strain will be selected from an existing database at the University of Stellenbosch. The strains will be cultured in liquid medium to midlog phase and when the appropriate growth phase is reached, the cultures will then be incubated in the presence or absence of sub-lethal concentration of INH for 24hrs, after which RNA will be extracted. Expression quantification of candidate genes will be done by quantitative Real-Time PCR and data analyses will be done by means of REST program using the delta-delta Ct equation.

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Chapter 2

Mycobacterium tuberculosis protective mechanisms against isoniazid

1. INTRODUCTION

There are several mechanisms that confer bacterial resistance to antibiotic, resistance can be intrinsic where the cell envelope permeability and efflux pumps plays a major role by controlling the amount of antibiotic allowed to penetrate the cell wall (21,41,56). Another resistance mechanism is acquisition of mutations through selective pressure of antibiotics or uptake of genetic information from other bacteria through horizontal transfer (6,114). *Mycobacterium tuberculosis* (*M.tuberculosis*) has refined these resistant mechanisms to ensure optimal survival.

The tubercle bacillus bases its pathogenicity on the ability to survive within the infected host by inhibiting phagolysosome biogenesis in microphages (46,78). A unique characteristic of *M.tuberculosis* is the ability to exist in a dormant state within infected tissue, where it can persist for several decades (39,64). This has been in part explained by the presence of the *M.tuberculosis* distinctive complex cell wall, which also plays a role in the growth and survival, low permeability to hydrophobic antibiotics, resistance to chemical injury, acid-base staining (12) and biofilm formation (95) of the bacterium (60,63).

Isoniazid (INH) is the most effective and specific anti-TB drug used for treatment of TB, as it is used for treatment of latent *M.tuberculosis* infection and prevention of active disease and

subsequent transmission (36,137). It enters the tubercle bacillus by passive diffusion and primarily targets mycolic acid biosynthesis (5,82,98). It is then activated by catalase peroxidase encoded by *katG* gene to produce acyl radical-INH-NAD adduct (55). This in turn blocks the InhA (NADH-dependent enoyl-[acyl-carrier-protein]) reductase located in FAS-II complex (113), resulting in the accumulation of saturated fatty acids which leads to killing of *M.tuberculosis* (138).

However the natural mutation rate of INH is higher than other anti-TB drugs, thus resistance of *M.tuberculosis* to INH occurs more frequently when compared to other drugs (111). As a result, global increase in the incidence of INH resistant *M.tuberculosis* strains have been observed over the years, with estimates of 10% in new cases and 27 % in previously treated cases (23). In addition, INH resistance has been associated with high level mutations in the *katG* gene and four other genes (*inhA*, *kasA*, *ndh* and *ahpC*) of *M.tuberculosis* (27). However, only 70-80% of INH resistant clinical isolates are phenotypically resistant (87). The inability to genotypically detect INH resistance in these isolates could be due to the presence of mutations in other unidentified genes or mechanisms.

This review will focus on the strategies adapted by *M.tuberculosis* to evade the toxic effect of INH by assessing not only (i) mechanical barrier but also mechanisms that are relevant to INH resistance such as (ii) target protection barrier and (iii) enzymatic barrier. A combination of these mechanisms might play a major role in the ability of *M.tuberculosis* to counteract INH toxicity.

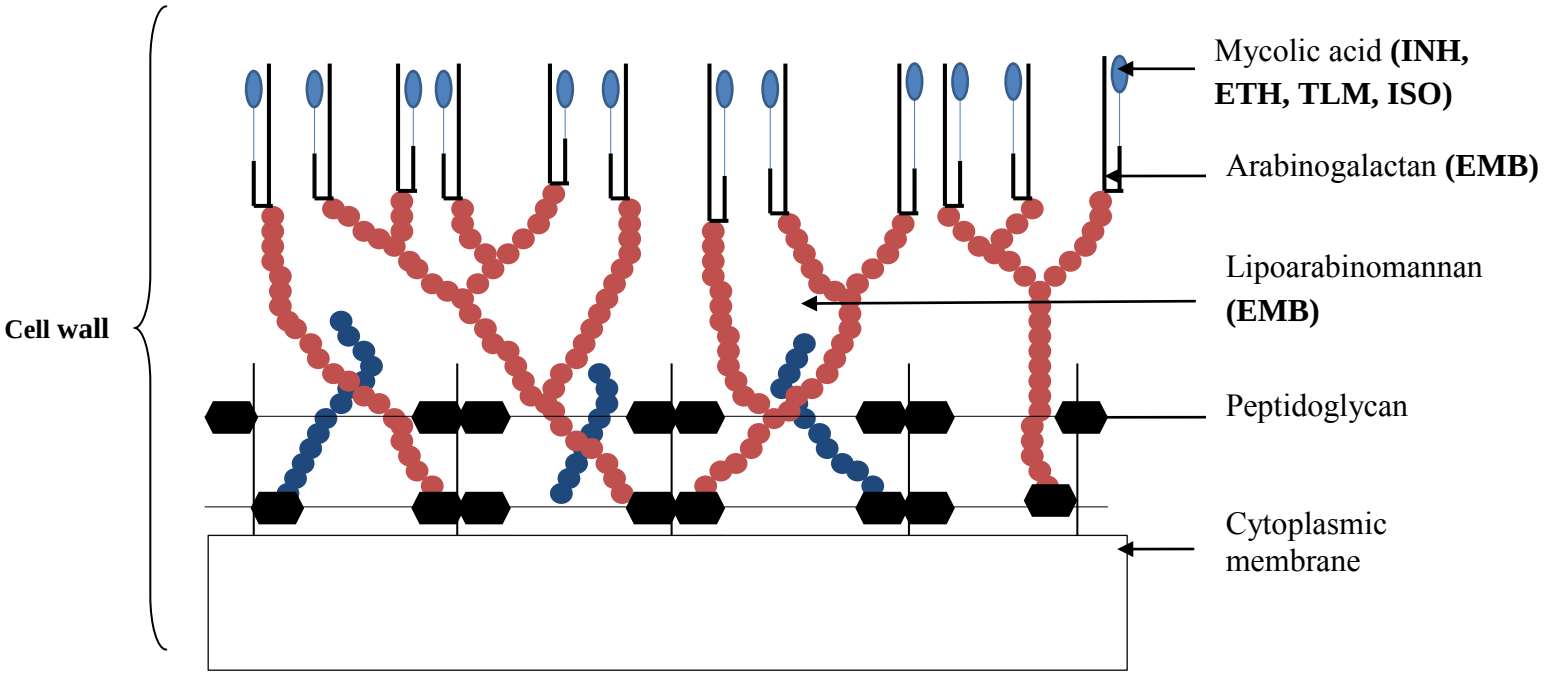
2. MECHANICAL BARRIER

Mechanical barrier is one of the protective strategies employed by *M.tuberculosis* against INH. This is done by modifying the membrane permeability through decrease of porins or in *M.tuberculosis* case by alteration of lipoglycans (51,93) which are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond found in the cell wall of gram-negative bacteria (4).

2.1. *M.tuberculosis* cell wall

M.tuberculosis has a very complex cell wall, composed of an insoluble matrix of cross-linked peptidoglycans linked to arabinogalactans (AG), esterified at the distal ends of mycolic acids (7,86). Additionally lipoarabinomannan (LAM) and mannosylated phosphatidyl inositol (PI) which are presented in the outer layer of the cell envelope are anchored in the cell membrane by lipidic position (11). An equally vital component of the mycobacterial cell wall is the mycolic acid, which constitutes up to 60% of the cell wall and is primarily accountable for the integrity and low permeability of the cell wall (7,17) which ensures that the tubercle bacillus is protected from the host's immune system and other toxic compounds (39). Consequently, mycolic acid synthesis has proven to be important for the pathogen's survival and growth (39). In addition, mycolic acids have a distinctive chemical nature and cell wall biosynthetic enzyme involved in their biosynthesis, formed targets for the design of anti-TB drugs such as (isoniazid (INH), isoxyl (ISO), thiolactomycin (TLM), ethambutol (EMB) and ethionamide (ETH)) (8,121) (Figure 1).

Figure 1: Representation of mycobacterial cell wall and location of the action site of anti-TB drugs targeting cell wall constituents



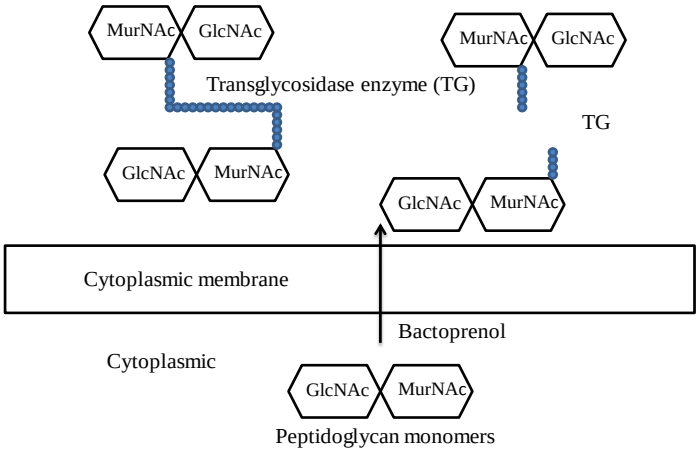
Legend to figure 1: INH = Isoniazid, ETH = Ethionamide, TLM = Thiolactomycin, ISO = Isoxyl, EMB = Ethambutol

2.1.1. Peptidoglycan

Peptidoglycan is a polymer that consists of identical alternating chains of *N*-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), cross linked to arabinogalactan (AG) (30). Synthesis of the peptidoglycan monomers takes place in the cytosol by sequential addition of $_{L}$-Ala, $_{D}$-Glu, meso-diaminopimelic acid and dipeptide D-Ala-D-Ala, catalyzed by MurA and MurC which is unique to mycobacteria (30,131). The monomers are then transported across the cytosolic membrane by a membrane carrier molecule, bactoprenol (9). The bactoprenol with the help of the transglycosidase enzyme

insert the peptidoglycan monomers to existing peptidoglycan which enables the bacteria to grow (Figure 2). Peptidoglycan structure is orientated in such a way that the mycobacteria cell wall retains its structural integrity and thus protected from degration by enzymes or antibiotics (97).

Figure 2: Mycobacterial biosynthesis of peptidoglycan

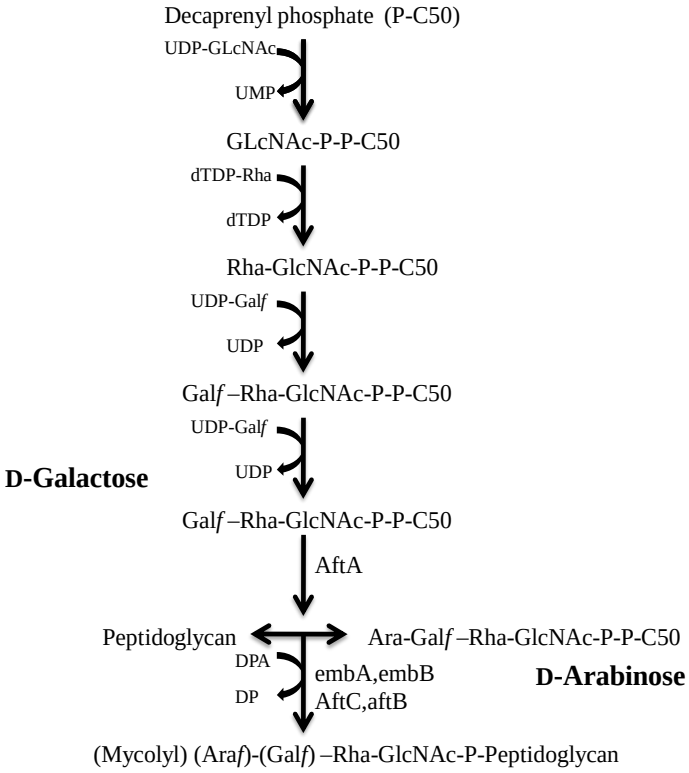


Legend to figure 2: GlcNAc = *N*-acetylglucosamine, MurNAc = N-acetylmuramic acid, TG = Transglycosidase enzyme

2.1.2. Arabinogalactan

Arabinogalactan (AG) is the second most dominant macromolecule in the mycobacterium cell wall, it is covalently linked to mycolic acids and peptidoglycan which then subsequently forms the mycolyl-arabinogalactan-peptidoglycan complex (mAGP) (30). The polymer is structurally unique in that is it composed of both D-arabinose (D-Ara*f*) and D-galactose (D-Gal*f*) in a furanose configuration which is rarely found in nature (11,30). The galactan is linked via the linker region (α-$_{L}$-Rha*p*-(1-3)-α-$_{D}$-GlcNAc-P) (30) to the muramic acid residues of peptidoglycan, while the arabinan chains are attached to the galactan core via the C-5 of some 6-linked Gal*f* units (30). The arabinan are further linked to the peptidoglycan via the diglycosylphosphoryl bridge ($_{L}$-Rha*p*-(1-3)-$_{D}$-GlcNAc-1-P) and mycolic acids are located in clusters of four on the terminal arabinofuranosyl units which are added by a set of unique arabinofuranosyltranferases (AraTs) (Figure 3) (106). Three of the AraTs which form part of the Emb protein (EmbA, EmbB and EmbC) have been shown to be present in both *M.avium* and *M.tuberculosis* (10,146), and the recently discovered proteins include AftA , AftB and AftC (1,123). The mAGP complex provides a linked network between the mycolic acid and the cytoplasmic membrane, thus making the mycobacterium cell wall difficult to permeate (30).

Figure 3: Mycobacterial biosynthesis of arabinogalactan



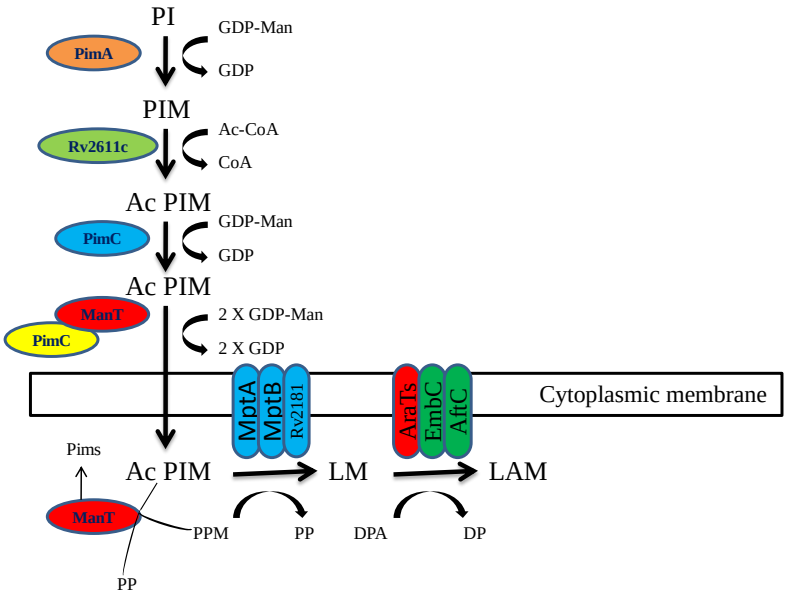
Legend to figure 3: P-C50 = Decaprenyl phosphate, Rha = rhamnose, GlcNAc = *N*-acetylglucosamine, Gal*F* = Galactofuran, Ara*F* = Arabinofuran, DPA = Decaprenyl-1-monophosphoarabinose, UDP

2.1.3. Lipoarabinomannan

Lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs) and lipomanna (LM) major lipoglycans found in the *M.tuberculosis* cell envelope (14,18). They are located at the exterior of the cell wall were they are attached to the plasma membrane by a phosphatidyl-*myo*-inositol anchor (18,92). Additionally, *M.tuberculosis* LAM has short mannose containing oligosaccharide "caps" which allows it to bind to the mannose receptors on macrophages (92).

LAM biosynthesis involves conversion of phosphatidylinositol (PI) to PIM via the mannosyltranferases (manT) catalyzed by PimA, PimB and PimC (47,66,68), further elongation of PIM leads to formation of the liner α1.6-linked mannan backbone of LM and LAM (147) (Figure 4). Although the function of LAM in the cell wall integrity is unclear, LAM displays pathology that is characteristic of TB, which include stimulation of macrophages to produce TNFα (18), nitric oxide and matrix metalloproteinases (18,24), inhibits activation of T-cell (24) and macrophage activation by interferon-γ (25). LAM is thought to be involved in the synergy between the Human Immuno Deficiency Virus (HIV)-TB , as it is capable of inducing transcriptional activation of HIV-1 (77).

Figure 4: Mycobacterial synthesis of lipoarabinomannan (13)



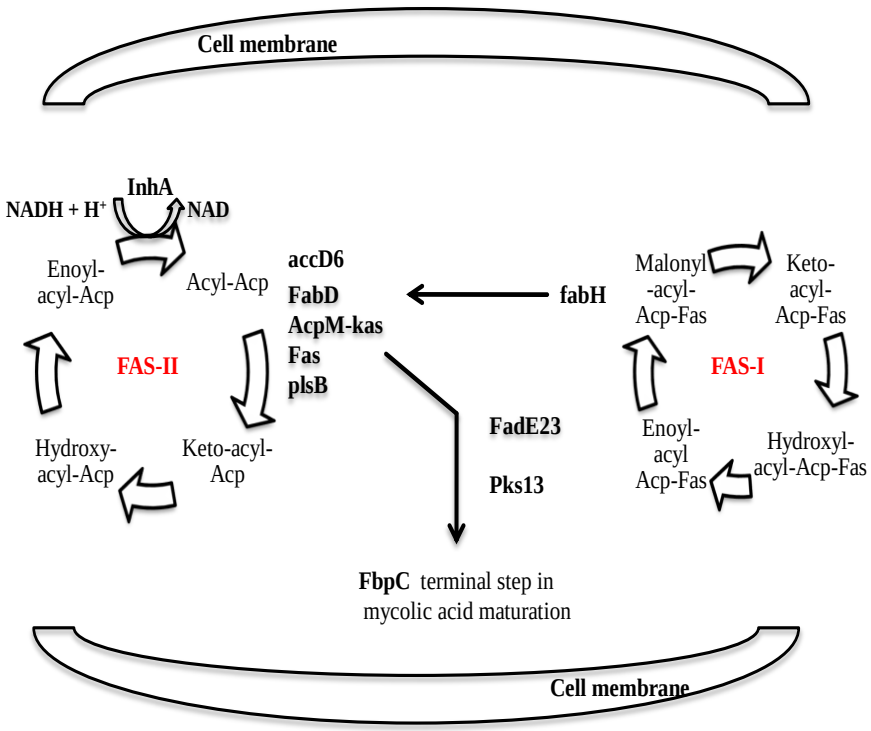
Legend to figure 4: PI = Phosphatidylinositol, PIM = PI mannoside, PPM = Polyprenylphosphomannose, LM = lipomannan, LAM = Lipoarabinomannan, ManTs = Mannosyltransferases

2.1.4. Mycolic acids

Mycolic acids are long-chain α-alkyl-β-hydroxy fatty acids (7). *M.tuberculosis* contains three families of mycolic acids: α-mycolic acids which constitute 70% of all the mycolic acids, methoxy and keto mycolic acid (7,143). Depending on the source, mycolic acids can vary in length of terminal, alkyl groups and number of methylene group (50). An example is the structural difference of α-mycolic acid from H37Rv and Canetti strains (115,143). Mycolic acid occur within the fluid matrix in the form of free trehalose dimycolate (TDM) and trehalose monomycolate (TMM) and both of these components are potent antigens and have been implicated in the pathogenesis of tuberculosis (43,57,58,75).

Mycolic acids are primarily synthesised by the fatty acid synthase (FAS) enzymatic system, found in mycobacteria as FAS-I and FAS-II complexes (12,122). The FAS-I complex is capable of synthesising and generating short chain fatty acid. The FAS-II multienzymatic complex further elongates the short chain fatty acids generated by FAS-I (12,122). Mycolic acids formation involves four unique pathways: (i) formation of short-chain acyl CoA of between C_{24}-C_{26} catalyzed by the FAS-I enzyme, (ii) synthesis of C_{58}-acyl-ACP (meromycolate) from C_{16}-acyl CoA by FAS-II system composed of multiple enzymatic reactions (iii) introduction of other functional groups to meromycolate by numerous cyclopropane synthases and (iv) final claisen condensation with C_{24}-acyl CoA of FAS-I catalysed by the polyketide synthase Pks13 and various mycolyltransferase processes to produce α-mycolic acids, methoxy- and keto mycolic acids (44,45,105,122), followed by the transfer of mycolic acids to arabinogalactan (133) (Figure 5).

Figure 5: Representation of Mycobacteria mycolic acid biosynthesis pathway (MAP)



Legend to figure 5: FAS = fatty acid synthase, InhA = NADH-dependent enoyl-[acyl-carrier-protein] reductase, AcpM = Acyl carrier protein, Kas = β-ketoacyl- ACP synthase, Fas = Probable fatty acid synthase, plsB = Possible acyltransferase, Acc = Acetyl /propionyl CoA carboxylase, Fad = Probable acyl-CoA dehydrogenise, Pks13 = Polyketide synthase, FbpC = Trehalose dimycolyl tranferase, NADH = nicotinamide adenine dinucleotide

2.2. Anti-TB drugs targeting *M.tuberculosis* cell wall

The distinctive structural composition of the *M.tuberculosis* cell wall is the site of action of a number of powerful anti-TB drugs and to a certain extent is responsible for resistance of most of these drugs including INH (17,119) (Figure 1). EMB a first-line anti-TB drug which targets both the AG and LAM biosynthesis is used in combination with other anti-Tb drugs to prevent mono-drug resistance (132). EMB inhibits numerous AraT's, which are encoded by the *embCAB* operon (135,147). Ethambutol resistance in *M.tuberculosis* strains has been associated with mutations found in the *embB*, *embA* and *embC* genes, most of which are found at codon embB306 and most recently also at codon embB406 and embB497 (59,135).

Thiolactomycin (TLM) is a unique thiolactone (37) that inhibits the FAS-II complex of many mycobacteria and as a consequence hinders synthesis of mycolic acid (125). TLM targets mycobacterial β-ketoacyl synthase III (*fabH*), which is an essential link between the FAS-I and FAS-II complexes (124). Another compound that was used as an anti-TB drug during the 1960s is a derivative from thiourea, Isoxy (ISO) (125). It has been reported that ISO inhibits both the mycolic acid and free fatty acids synthesis of all slow and fast growing *Mycobacterium* species (124), especially that of *Mycobacterium bovis BCG* (67). In addition, it was later demonstrated that ISO also inhibits oleic acid biosynthesis by blocking the stearoyl co-enzyme A (CoA) and desaturase DesA3 involved in lipid metabolism in the mycobacterial cell envelope (103).

Ethionamide (ETH) is a structural analogue of INH (118). Both these drugs are prodrugs and require intracellular activation to acquire bacteriotoxicity (3). Monooxygenase encoded by the *EthA* gene activates ETH (38). Subsequent to activation ETH inhibit mycolic synthesis by

targeting the enoyl acyl carrier protein (InhA) encoded by the *InhA* gene (3). Furthermore, isolates resistant to ETH have low level INH resistance which results from higher frequency of mutation found in the *katG* gene when compared to those found in *InhA* gene (142,150).

2.3. Multidrug efflux pumps

Intracellular concentration of drug mycobacterial cell is generally due to a balance between influx which is provided by the bacterial unique lipid-rich cell wall and efflux by active efflux pumps (41,93,140). Multidrug efflux pumps appears to be one of the major mechanism of resistance in wide variety of bacteria and has been shown to aid *M.tuberculosis* with intrinsic resistance to various anti-TB drugs (41,93).

Drug transporters can be divided in two groups: those that use an ion gradient such as sodium or proton ions across the membrane and extrude drugs against an electrochemical gradient (31), included in this group are major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE), resistance-nodulation-cell division (RND) and small multidrug resistance (SMR) transporters. The second class uses free energy in a form of ATP to pump drugs out of the cell and mainly include the ATP-banding cassette (ABC) families (69,79).

Drug efflux-related mechanisms in INH resistance have been described in a number of mycobacteria (28,73,74,100). For instance the *M.tuberculosis* mycobacterial membrane protein (MmpL7) catalyzes the export of phthiocerol dimycocerosate (PDIM) and overexpression of the *MmpL7* gene in *M.smegmatis* has been shown to present high-level

INH resistance, whereas a decrease in resistance level was observed in the presence of efflux inhibitors (100). This result was explained by the observed association of *MmpL7* in the energy-dependent efflux of INH (100). The *ini* region has a total of four genes, three of which are organized as an operon (*iniA,iniB and iniC*) with the *Rv*0340 gene located of upstream the *iniBAC* operon (2). Analysis of *iniA* gene in *M.tuberculosis* through knockout studies have proven that the *iniA* gene is essential for the MDR-pump like mechanism that confers tolerance to both INH and ETH (29). Several MFS efflux pumps have been shown to be regulated in *M.tuberculosis* through gene expression studies after INH induction, these include (*efpA, pstB*, *Rv1258c* and *Rv1410c*) and ABC transporters (*Rv1819c* and *Rv2136c*) (42,61,108,141,145) (Table 1).

Collectively these results suggest that efflux related mechanisms may influence the intracellular concentration of activated INH in the mycobacterium cell and therefore might be involved in *M.tuberculosis* INH resistant isolates which do not have genomic mutations associated with the drug.

Table 1: Efflux pump genes and transporters regulated by INH in *M.tuberculosis*

Efflux gene	**Transporter**	**Function**	**Protein product**	**reference**
MmpL7	RND	Export of antibiotic	transmembrane transport protein	(35,100)
pstB	ABC	Active import of inorganic phosphate and export of drugs	Phosphate-transport ATP-binding protein	(117)
iniA	membrane protein	Drug transport	INH inducible protein iniA	(2,29)
iniB	membrane protein	Drug transport	INH inducible protein iniB	(2)
iniC	membrane protein	Transcriptional mechanism	INH inducible protein iniC	(2)
efpA	MFS	Export of drugs	Integral membrane efflux protein	(76)
Rv1747	ABC	Transport of drug across the membrane.	conserved transmembrane ATP-binding protein	(62,85)
Rv1410c	MFS	Export of drugs	Aminoglycosides/tetracycline-transport integral membrane protein	(110)
Rv1258c	MFS	Export of drugs	Conserved membrane protein	(31)
Rv1819c	ABC	Export of drugs	probable drug-transport transmembrane ATP-binding protein	(16)
Rv2136c	ABC	Unknown	Possible conserved transmembrane protein	(16)
Rv2459	MFS	Transport of drug across the membrane.	Probable conserved integral membrane transport protein	(49)

3. TARGET PROTECTION BARRIER

3.1 Genomic alteration

The mycolic acid pathway (MAP) involves numerous reactions of multiple genes, yet only few and essential genes have undergone alteration due to INH treatment (133,134). It seems that the tubercle bacilli has evolve a survival strategy to protect itself from INH by acquiring mutations at selected multiple genomic positions that strongly decrease the affinity of the drug to the target site.

Mycobacterium catalase peroxidase enzyme, encoded by *katG* gene serves as both a catalase to protect *M.tuberculosis* from reactive oxygen species produced by the microphage and also functions as a peroxidase, to activate INH into a isonicotinonyl acyl radical (144). Mutations in the peroxidase are a major mechanism of INH resistance in *M.tuberculosis* clinical isolates (148), approximately 130 mutations with minimum inhibitory concentration (MIC) ranging from 0.2 and 256μg/ml associated with INH resistance (54,107,149). Mutations at codon Ser315Thr are the most common found in 50-90% of INH resistance isolates and associated with high level INH resistance (137). Complete deletion of *katG* gene is rare (15,127) and if it ever occurs it was only found in less than 5% of the INH resistance isolates (54). Mutations in *katG* results in reduced peroxidise activity resulting in the inability to activate INH (52,109), this in turn causes poor formation of the INH-NAD adduct preventing it from binding to InhA (26).

A direct link between the synthesis of mycolic acids and INH was first described in 1994 by the identification of InhA as a target for INH (128). The *inhA* gene was shown to have a non-

synonymous mutation at codon Ser94Ala located in the binding pocket of NADH which reduces binding of the activated INH radical to NAD (3) and consequently disturbs binding of INH-NAD adduct to InhA (8,33). Other mutations located in the binding pocket of *inhA* gene include (lle16Thr, lle21Thr, lle21Val, lle47Thr, Val78Ala, lle95Pro and lle194Thr) (120,137). However, INH resistance in *M.tuberculosis* clinical isolates have been commonly associated with *inhA* promoter mutations which are present at positions -8(T-G/A), -15(C-T), -16(A-G), -17(G-T) and -24(G-T) of which -15(C-T) is the most prominent (72,120). The consequence of these promoter mutations is overexpression of *inhA* gene, which leads to low level INH resistance (70).

Another genomic alteration that confers resistance to INH is mutations found in *ndh* gene (22,109). This was first postulated in 1998 in the INH and ETH resistance isolates of *M.smegmatis* where reduced activity of NADH dehydrogenase due to mutations resulted in coresistance of INH and ETH (83). The *ndh* gene encodes type II NADH dehydrogenase (Ndh) found in the *M.tuberculosis* genome (83). To date two distinct mutations at codon T110A and R268H have been identified in the *ndh* gene of INH resistance *M.tuberculosis* clinical isolates (71,83) and mutations in this gene diminishes Ndh activity causing accumulations of nicotinamide adenine dinucleotide NAD(H) and reduction of NAD^+ (71). This defect increases NADH intracellular level which competitively inhibit the binding of INH-NAD adduct to InhA and ultimately leads to co-resistance of INH and ETH (139).

Alkyl hydroperoxide reductase enzyme (AhpC) is encoded by the *ahpC* gene, a member of peroxiredoxins (48). The enzyme protects *M.tuberculosis* from reactive oxygen species and is vital for oxidative stress response (39,48). Regulation of this protein takes place at the bacterial *oxyR* gene in most mycobacteria and appears to be linked to the altered expression of the *ahpC* gene (34,96). Mutations in *oxyR-ahpC* locus increase the expression of AhpC in

katG deficient INH resistant strains (130,141,145) which has been observed as a compensatory mechanism for the loss of catalase peroxidase in the strain (80). Therefore several aspects of oxidative stress response, including regulatory factors have been associated with inherent susceptibility and acquired resistance to INH (20,32).

kasA gene which encodes ketoacyl acyl carrier protein synthase (kas) forms part of a five genes (*kasA*, *kasB*, *accd6*, *acpM* and *fabD*) operon which is vital for extension of mycolate from FAS-I during the synthesis of mycolic acid (129), although the role of this protein in INH resistant is still unclear, several mutations have been reported in the gene (104,129). The MAP is one of the important pathways involved in the survival of mycobacteria, multiple genomic alterations induced by INH treatment in this pathway might be a survival mechanism that the bacterium has evolved to shield itself against the toxicity of the drug

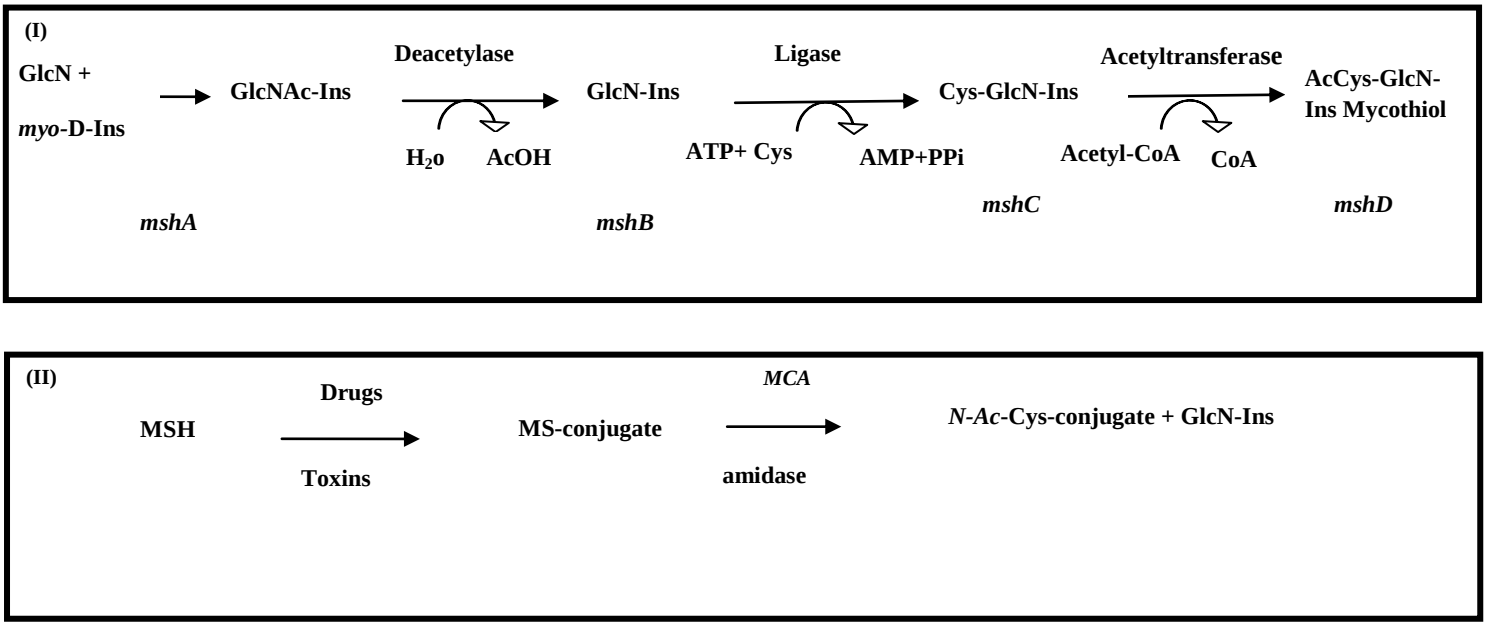
3.2. Mycothiol deficient

Mycothiol (MSH) is a major thiol in mycobacteria and it is composed of 1-O-(2-amino-2-deoxy-α-D-glucopyranosyl)-D-myo-isotol (116). It plays crucial role in detoxification of a variety of thiol-reactive agents (116). Biosynthesis of MSH in *M.tuberculosis* proceeds through a four-step pathway which involve the following seven genes: (i) glycosyltransferase *mshA* (91), (ii) deacetylase *mshB* (89), (iii) ligase *mshC* (116), (iv) mycothiol synthase *mshD* (65), (v) NAD/MSH-dependent formaldehyde dehydrogenase *mscR* (84), (vi) mycothione reductase *mtr* and (vii) mycothiol S-conjugate amidase *mca* (88) (figure 5).

A study conducted in 2003 has shown that *M.tuberculosis* mutants lacking mshB activity showed a twofold increase in INH resistance and *M.smegmatis* mutants which had transposes

in either mshA, mshC or mshD were also found to be resistance to INH (19). On the contrary, *M.smegmatis* mutants lacking MshB activity were found to be sensitive to INH (112). More recently mycothiol have been shown to play a role in the activation of ETH by the *ethA*-encoded mono-oxygenase and are therefore essential for ETH susceptibility in *M.tuberculosis* (136). Although further studies are needed to elucidate the initial step of MSH biosynthesis it might be that MSH-dependent detoxification in combination with other mechanism facilitate the removal of INH from the bacterial cell.

Figure 3: (I) Mycothiol biosynthesis pathway (90) and (II) MSH-Mediated Detoxification (88)



Legend of figure 3: Ac = Acetyl, CoA = coenzyme A, GlcN = glucosamine, MCA = mycothiol-*S*-conjugate amidase, MSH = Mycothiol, AcCys = mercarpturic acid

4. ENZYMATIC BARRIER

Antibiotic modification by *M.tuberculosis* may be an additional way that the bacteria acquire resistance to INH, in this case the tubercle bacillus employs *N*-acetyltranferase to modify the drug.

4.1. Over production of arylamine *N*-acetyltranferase

Arylamine *N*-acetyltranferase (NAT) exists in humans as two polymorphic isoforms (NAT1 and NAT2) (53). The NAT2, a hepatic phase II drug-metabolizing enzyme is responsible for the acetylation of INH in the liver via the acetyl coenzyme A (acetyl-CoA) (126). After acetylation, acetyl-iso-isoniazid (Ac-INH) is formed and it is further hydrolyzed into iso-nicotinic and mono-acetyl hydrazine (MAH) (40,99). Single-nucleotide polymorphisms (SNPs) of NAT2 determines pharmacogenetic variations in humans (81), and can be used to distinguish between slow, intermediate and fast acetylator phenotypes for INH (53). Subsequent to INH treatment the NAT2 slow acetylators excrete large amounts of INH relative to NAT2 fast acetylators (99). The common cause of NAT2 slow phenotype has been explained by NAT2 decreased expression which bears amino acid substitution at codon I114T, resulting from the T to C transition found in *NAT2*5* alleles (94).

A study done in 2001 showed that the *nat* gene encodes NAT in both *M.tuberculosis* and *M.smegmatis*, The investigators also reported that overexpression of *M.tuberculosis nat* gene in *M.smegmatis* resistant bacillus increased resistance to INH threefold (102). In addition, deletion of *nat* in the bacillus illustrated increased sensitivity to INH (101). Inactivation of INH by the *nat* gene in *M.tuberculosis*, results in the acetylation of the nitrogen group of INH

and doing so it inhibits INH activation by catalase peroxidase enzyme, rendering the drug therapeutically inactive (101,102). These results confirm that reduced activity of NAT shields *M.tuberculosis* from drug as a result of lower activity or mutation in genes encoding *N*-acetyltranferase.

5. CONCLUDING REMARKS

Resistance of *M.tuberculosis* to INH is mainly due to various genomic mutations in the drug main target (MAP). However, there are *M.tuberculosis* clinical isolates resistant to INH that does not present these mutations, which clearly indicates that *M.tuberculosis* utilizes additional resistant mechanism for INH. Intrinsic resistant is one of the major resistant mechanisms that a broad variety of bacteria employ and it mainly involves the balance between influx provided by the lipid rich membrane and efflux presented by the efflux pumps. INH enters the cell by passive diffusion which allows the drug to escape the cell wall barrier. Alteration in the gene responsible for the activation of the drug insures that the bacilli is protected from the toxicity of the drug which can be viewed as a second line of protection after penetration of the bacterial complex lipid-thick cell wall. In addition, the bacterium can regulate the intracellular regulation of INH by activating the efflux pumps thereby causing the extrusion of the activated drug from the bacterium. In doing so the bacteria survives and go on further to acquire resistance. Therefore, *M.tuberculosis* protection against INH might involve a combination of mechanical barrier (cell wall and efflux pumps), target protection barrier (genomic alteration) and enzymatic barrier (antibiotic modification).

Further understanding on how these mechanisms interlink to serve as a protective mechanism to INH toxicity in *M.tuberculosis* may help to improve existing anti-TB drugs and provide a novel approach to development of new anti-TB drugs.

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Chapter 3

MATERIALS AND METHODS

3.1 Selection of candidate genes by literature searches

Candidate genes were selected from reported literature and included genes involved in the mycolic acid synthesis pathway (targeted by INH) and those involved in drug transport (1,3,4,9,12).

3.2 Selection of *M.tuberculosis* clinical strains

M.tuberculosis clinical isolates from the Beijing cluster 208 INH susceptible (K636) and INH mono-resistant strains (R55) were selected from a longitudinal reference database with well characterized isolates which is maintained at the University of Stellenbosch. Phenotypic characterization of these isolates included drug susceptibility testing in BACTEC 12B medium (Becton Dickinson, USA) at the critical concentration of 0.1µg/ml INH. Subsequent minimum inhibitory concentration (MIC) determination for the susceptible (K636) and the resistant (R55) isolates showed MIC's of 0.1 µg/ml and 4µg/ml INH, respectively. In addition, these isolates were genotypically characterized by spoliogotyping (5), *IS6110* DNA fingerprinting (11) using internationally standardized methods (Table 4.1).

3.2.1 Single colonies isolation

To select single colony forming units, undiluted glycerol stocks of the selected INH susceptible (K636) and INH mono-resistant (R55) strains were inoculated into Middlebrook 7H10 agar (supplemented with 0.2 % (v/v) glycerol, oleic acid-dextrose-catalase 10% OADC) containing (0.01, 0.1 and 4 µg/ml) concentration of INH. Control agar plates did not contain any INH. The plates were incubated at 37°C for 21 days to allow for colony formation. Single colonies were picked and subsequently cultured in 20 ml of enriched 7H9 Middlebrook medium (supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween 80 and 10% OADC) and incubated at 37 °C without shaking for 14 days. At this stage 10ml of the culture was stored in 1 ml aliquots at -80 °C until required.

3.2.2 DNA isolation

Crude DNA was isolated by boiling an 1ml aliquot of the cultured single colony inoculum of each isolate in a 1.5 ml eppendorf tube (Merck, New Jersey, USA) for 30 minutes at 80°C. The crude DNA templates were then stored at -20°C prior to DNA sequencing.

3.2.3 Primer design and mutation screening

Primers were designed for amplification using Primer software 3 version 0.2 (Whitehead Scientific) and sequencing of known INH resistance causing gene mutations (*katG, inhA, kasA, ndh, ahpC*) (Table3.1). Gene sequences were searched on (http://genolist.pasteur.fr/TubercuList).

3.2.4 PCR amplification

DNA amplifications were done in 25 µl reaction volumes. The PCR master-mix consisted of 5µl 5X Q-solution, 2.5µl 10X reaction buffer, 2 µl $MgCl_2$ (25mM), 4 µl deoxyribonucleotide triphosphates (dNTPs) (0.2mM of each dNTP), 0.5 µl Forward primer (50pM), 0.5 µl Reverse primer (50pM), 0.125 µl Hotstar Taq polymerase (5 units/µ), 1.25 µl of the crude DNA template, and 7.85 dH_2O to make up a final volume of 25 µl. The reaction mixtures were heated in the thermal cycler (GeneAmp PCR System 2400, Applied Biosystems, Foster City, CA, USA). Successful amplification of the DNA fragment was assessed by visualization on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) stained with ethidium bromide.

3.2.5 Sequencing

DNA sequencing was done on ABI3730xl DNA analyzer at the Central Analytic Facility of Stellenbosch University. Sequences were then compared with the whole genome sequence of *M.tuberculosis* H37Rv reference (http://genolist.pasteur.fr/TubercuList) to determine if mutations are present in the candidate genes from different *M. tuberculosis* isolates.

Table 3.1: PCR primers and conditions used to amplify genes associated with INH resistance

Region	*Forward / Reverse primers*	*Tm (°C)*	*Amplification fragment (bp)*	*Reference*
katG				
	5' CAGAAACCACCACCGGAGCC '3	59	945	(9)
	5' GCTGGTGATCGCGTCCTTAC'3			
	5'TGGCCGCGGCGGTCGACATT'3	64	804	(9)
	5'TCGGGGTCGTTGACCTCCCA'3			
	5'CCGACGATGCTGGCCACTGA'3	62	997	(9)
	5'GACCTCGACAAGCGCCCGCA'3			
InhA gene	5'CGGGCAACAAGCTCGACGGG'3	64	169	(9)
	5'GGGTTCATGATCGGCAGGAG'3			
Inh A promoter	5'CGCAGCCAGGGCCTCGCTG'3	60	246	(9)
	5'CTCCGGTAACCAGGACTGA'3			
ahpC promoter	5'GCTGATTGTCCGAGAGCATCG'3	60	701	(10)
	5'GGTCGCGTAGGCAGTGCCCC'3			
ahpC intergenic	5'CCGATGAGAGCGGTGAGCTG'3	66	236	(10)
	5'ACCACTGCTTTGCCGCCACC'3			
ndh	5'ATCACCACCGCCGCTGAAGC'3	65	1135	(10)
	5'GTTCGGGTACCCGGGAATG'3			
	5'CATTCCCGGGTACCCGAAC'3	65	535	(10)
	5'GTCGACCGTTTTGGCGTTGG'3			
kasA	5'GTTCAGGCGAGGCTTGAG'3	50	1293	(10)
	5'GCGATGTCGTGCTTCAGTAA'3			

3.3 *M.tuberculosis* culture conditions and drug treatment

M.tuberculosis Beijing cluster 208 fully susceptible (K636) and INH mono-resistance (R55) strains were grown on Lowenstein-Jensen (LJ) slants with continuous aeration for approximately three weeks. Colonies were scraped from the LJ slants and inoculated into 5 ml 7H9 Middlebrook medium (supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween 80 and 10% ADC) and incubated at 37 °C without shaking, until an OD_{600} of 0.6-0.8 was reached. Thereafter these starter cultures were inspected for contamination by Ziel-Neelsen gram staining and culture on blood agar plates. At this stage 1ml aliquots were stored at -80 °C after which a sub-starter culture was setup by inoculating 800 µl freezer stock into 80 ml 7H9 media and incubated at 37°C without shaking, until an OD_{600} of 0.6-0.8 was reached.

Two biological replicate experiments were performed (to assess biological measurements for two independent experiments done on different days) and 2 technical replica experiments (to measure each biological sample on the same day) for each strain. The 80 ml liquid culture was divided into 2 x 40 ml cultures; INH (0.1 µg/ml) was added to one 40ml culture, while the remaining 40 ml was untreated and served as the control. Both cultures were subsequently incubated at 37°C for 24 hours.

3.4 RNA isolation and purification

Subsequent to the 24 hours incubation, 200ml of Guanidinium thiocyanate (5M) (GITC) (Sigma-Aldrich, St. Louis, USA) solution (5M GITC, 0.5% sodium *N*- lauroyl sarcosine, 0.1M β-mercaptoethanol, 12.5ml of 1M sodium citrate pH7.0 and 1% Tween 80) made up to

500ml with RNase free water was immediately added to each 40 ml culture. Bacterial cells were harvested by centrifugation (3000rpm for 15 min, 20°C) and the supernatant was discarded. The pellet was resuspended in 1 ml TRIzol (Sigma-Aldrich, St. Louis, USA). The TRIzol suspension was transferred to a 2 ml lysing matrix tube containing silica beads (IEPSA) and ribolyzed for 45 seconds at 6 Watts for 3 cycles and cooled on ice for 1 minute between pulses. The samples were centrifuged at 12000 rpm for 10 min and the TRIzol solution above the bed of the beads was transferred to a 2 ml phase lock gel tube (Eppendorf, Hamburg 22331, Germany) containing 300µl chloroform (Sigma-Aldrich, St. Louis, USA), and vigorously mixed. Samples were incubated at room temperature for 5 min to allow phase separation. The aqueous layer was recovered by centrifugation at 12000 rpm for 10 min and the top aqueous layer was transferred into a new 1.5 ml eppendorf tube. The RNA was precipitated with the addition of an equal volume of isopropanol (Merck, Darmstadt, Germany). This was followed by incubation at room temperature for 5 3.7min and then incubation at 4°C overnight.

Crude total RNA was collected by centrifugation at 12000 x g, 30 min at 4°C and the pellet was washed with 70% ethanol (Merck, Darmstadt, Germany), air-dried and re-dissolved to final concentration 70 µl RNase-free water. Possible DNA contamination was removed by digesting a 10µl aliquot of total RNA with 1 unit of RQ1 DNase (Promega) at 37°C for 30 min. To inactivate the digestion reaction DNase treated RNA was diluted to a final volume of 200 µl with RNase-free water and equal volume of phenol:chloroform (4:1) (Merck, Darmstadt, Germany) was added to the RNA, gently mixed and set on ice for 10min. The top layer was collected by centrifugation at 12000 rpm for 10 min at room temperature and transferred to a new tube. Subsequent addition of 0.1 volumes of 3M Sodium acetate pH 5.2

and 2.5 volume of 100% ethanol, followed by incubation at 4°C overnight. Purified RNA was pelleted by centrifugation (30 min, 12000 x g, 4°C), washed with 70% ethanol and collected by centrifugation at 12000 x g, for 10 min at 4°C. Ethanol was removed and the purified RNA was air-dried and re-dissolved in 20µl RNAse free-water and stored at -80°C until further use. The total RNA quality and quantity was assessed by various methods shown in figure 4.1.

3.5 cDNA synthesis and primer design for selected candidate genes

Purified RNA was used to synthesize cDNA according to manufacturer's instructions Quantitect Reverse Transcriptase kit (Southern Cross biotechnology). One microgram RNA was mixed with 2 µl genomic DNA Whipeout Buffer and RNase-free water to a final volume of 14 µl and incubated for 2 minutes at 42°C. After incubation Quantiscript Reverse Transcriptase, Quantiscript RT buffer, and RT Primer Mix was added to the RNA mix and incubated for 15 minutes at 42°C. After incubation the mix was incubated for 3 minutes at 95ºC to inactivate the Quantiscript Reverse Transcriptase. Gene sequences for candidate genes were searched on (http://genolist.pasteur.fr/TubercuList) and subsequent primers were designed using Primer software 3 version 0.2 (Whitehead Scientific) (Table 3.2).

3.6 Quantitative Real-Time PCR (QRT-PCR)

A volume of 1µl of each primer pair (10µM) (Table 4.3), 2µl LightCycler FastStart DNA masterplus SYBR green Mix (Roche Diagnostics, Indianapolis, USA 0001), 2µl of diluted cDNA (1:10) and RNase free water was added to the master mix in a 20 µl reaction. QRT-

PCR was performed using LightCycler 2.0 system (Roche Diagnostics). A four step PCR parameter protocol was used: denaturation program (95°C for 10 min), amplification and quantification program repeated for 45 cycles (95°C for 10 s, 59°C for 15 s, 72°C for 6 s), melting curve program (95°C for 0 s, 60°C for 15 s, 95 °C with a heating rate of 0.1°C /s) and cooling program of 40°C or 30s. Each QRT-PCR experiment was done on duplicate biological samples that were each assayed in duplicate.

3.7 Data analysis

The gene expression of target genes was determined by using the delta-delta Ct calculation in which the relative abundance of mRNA of the target gene was normalised relative to the level of reference RNA transcript (16srRNA and *Ftsz*) (6,7). Data analyses were done according to the delta-delta Ct equation $R=2^{-(\Delta CT \text{ sample } -\Delta CT \text{ control})}$. Only experiments with a standard deviation of <0.5 were included for analysis by Relative Expression Software Tool - 384 (REST-384©) which determines whether there is a significant difference between two groups of samples, taking into account issues of reaction efficiency and reference genes normalization. The software identifies significant fold changes and assigns significance with a significance level of 5% (8). In this study increase or decrease of at least fivefold in gene expression was considered significant.

Table 3.2: Primers used for QRT-PCR analysis

Gene	*Forward/Reverse primers*	*Size (bp)*	*Tm(^oC)*	*Reference*
katG	5' GAG CCC GAT GAG GTC TAT TG 3'	120	60	(9)
	5' ACG TAG ATC AGC CCC ATC TG 3'			
furA	5' AAC CAC CAT CAC ATC GTC TG 3'	115	60	Current study
	5' GTC ACC CAG GAA GCC GTT AT 3'			
ahpC	5' TCA GCA AGC TCA ATG ACG AG 3'	120	60	(9)
	5' ATC GGG AAG GGT AAC GTT TT 3'			
ndh	5' CTT ATT TCG GCA ACG ACC AT 3'	100	60	Current study
	5' CGA CAA CGG TGA ATG TCA GT 3'			
dfrA	5' GCT GAG GTT GTC GGT TCA CT 3'	120	60	Current study
	5' CGA CCT CGG TAA CCT CAC AT 3'			
InhA	5' ATC CAC ATC TCG GCG TAT TC 3'	100	60	(9)
	5' ACC GTC ATC CAG TTG TAG GC 3'			
acpM	5' AGG ACA AGT ACG GCG TCA AG 3'	120	60	(9)
	5' GGG GTT CTC CGA CTC AAT CCT 3'			
kasA	5' CCG ACC CTG AAC TAC GAG AC 3'	100	60	(9)
	5' AAC CCG AAC GAG TTG TTG AC 3'			
fas	5' CGA TGC AGA CGA TGT ACC AG 3'	120	60	Current study
	5' CCG ACG TAG GAC TGA ACC AC 3'			
plsB	5' GCT GAA GCC AAT TTT GTG GT 3'	100	60	Current study
	5' GTC ACC GTC GGC ATA GAA GT 3'			
fabD	5' CAA ACC GAG GGA ATG TTG TC 3'	100	60	Current study
	5' CGG TGT CGG TGA TCT CCT C 3'			
fadE	5' AGC CAG CGA CAA CGA CTA TT 3'	120	60	(9)
	5' TTC ACA TAC GGG ACG ACG TA 3'			
accD6	5' TCG AAG ACC AGA GTC CCA TC 3'	120	60	(9)
	5' AGA TCT GCG GGA TGT AGC C 3'			
Pks13	5' CAC GAC GTC GAA TAC TGG AA 3'	120	60	Current study
	5' AGC TCC AGG AAG GTG GTG T 3'			
fbpC	5' ATA TCT GCA GGT GCC ATC C 3'	120	60	(9)
	5' ATG TCC CAG CCG TTG TAG TC 3'			
efpA	5' TAG GTT TCA TCC CGT TCG TC 3'	115	60	(2)
	5' CAC GGT GCA TGA AAA ATG AG 3'			
iniA	5' AAG ATG ATC CAG CGT CTG CT 3'	173	60	(2)
	5'TTG ACC TGG CTC AGG ATA CC 3'			
iniB	5' GCT AGC CAG ATC GGT GTC TC 3'	171	60	(2)
	5' CGA CAG ATG AGG CAT AGC AG 3'			
Mmpl7	5' TGA AAT ACG GAA GCC TGG TC 3'	197	60	(2)
	5' GAG GTA AGA GGC CAG CAC AC 3'			
pstB	5' GTT CCC GAT GTC AAT CAT GG 3'	166	60	(2)
	5' ACC ACC AGA GAG TCG AAA CG 3'			
Rv1258c	5` GGT ATG CCG TGT TGG CTA TC 3`	188	60	(2)
	5` CCG CGT CTG TAT CAC GTA GTT 3`			
Rv1747	5' TCT GGA GCT GTT CGT TGA TG 3'	192	60	(2)
	5' ACC CAG GAC ATC TGG TCA AG 3'			
emrB	5' TTC GAC TAC ATG GGC CTC TT 3'	183	60	(2)
	5' TAT GAG CGG ATG TTC TGT GC 3'			
Rv1819c	5' CCG TCG TAG ATG GAA AAC AGG TG 3'	115	60	Current study
	5' CGT CTC CTC ACA AAC AGA AAT CCT 3'			

Reference

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Chapter 4

RESULTS

Table 4.1, illustrates genotypic and phenotypic characteristics of the two *Mycobacterium tuberculosis* Beijing clinical isolates used in this study.

Table 4.1: Genotypic and Phenotypic characteristic of selected strains

Isolates	Beijing cluster	Spoligotype pattern	IS6110 RFLP	MIC (µg/ml)	Phenotypic resistance profile	Genotypic resistance profile
K636	208			0.1	INH susceptible	No mutations
R55	208			4	INH-mono resistant	No mutations

Legend to table 4.1: MIC = Minimum inhibitory concentration, INH = Isoniazid

4.1 Assessment of RNA integrity and quantity

Figure 4.1, shows a representative of three quality controls measures: The integrity and quantity of RNA was verified by Experion software version 2.01 where the 16S and 23S rRNA appeared as sharp bends on a virtual gel (Figure 4A and 4B). The quantity and purity of the RNA was assessed spectrometrically by the nanodrop (Inqaba Technologies) at A_{260}/A_{280} ratio of between 1.8 and 2.1 (Figure 4C), only extracts with absorbance (A_{260}/A_{280}) of between 1.8 and 2.1 were used in subsequent experiment. In addition, the extracted total RNA was also examined for possible DNA contamination on a non-denaturing 1% agarose gels (Figure 4D).

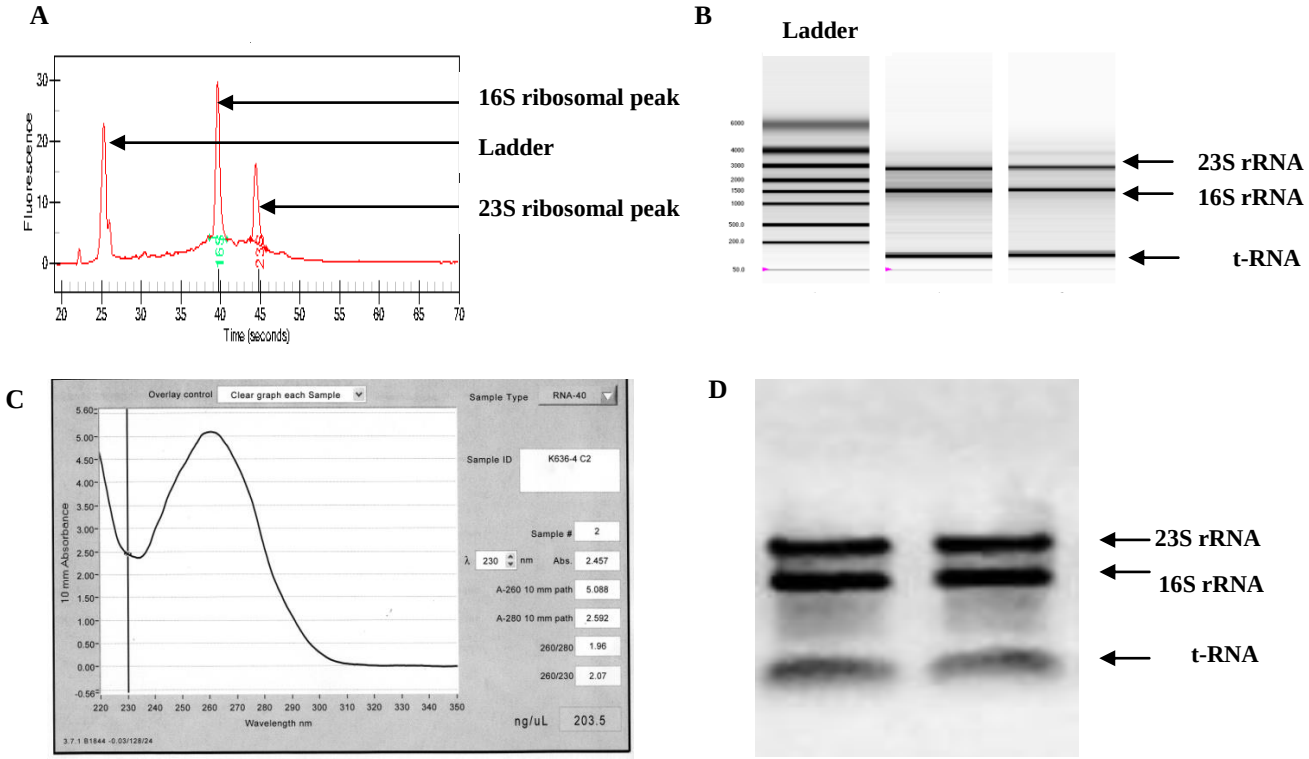

Figure 4.1: Representative quantification of RNA on different systems

Legend to figure 4.1: (A) virtual gel profile from Experion software indicating the ladder peak, and 16S and 23S ribosomal peaks that is indicative of prokaryotic RNA profile, (**B)** Experion virtual gel profile showing the ladder and RNA bands**,** (**C)** RNA absorbance measurement from the NanoDrop indicating the concentration (ng/µl), 260/280 and 230/280 readings, (**D)** gel image from 1% agarose gel showing 23S, 16S and t RNA bands

4.2 Literature search for candidate genes

Twenty four candidate genes were selected from the literature. The selection criteria were as follows: Fifteen of the candidate genes formed part of the mycolic acid pathway (MAP), the main target of INH and 9 drug transport genes shown previously to be induced by INH exposure (Table 4.2).

Table 4.2: Selected candidate genes induced by INH based on literature searches.

Genes	*Function*	*Product*	*Reference*
Mycolic acid pathway genes			
katG	Catalase-peroxidase activity	Catalase-peroxidase-peroxynitritase	(20,45)
furA	Global negative controlling element	Ferric uptake regulation protein	(25,30,44)
ahpC	Oxidative stress response	Alkyl hydroperoxide reductase C protein	(16)
ndh	Electron transfer	Probable NADH dehydrogenase	(22,38)
dfrA	Intermediary metabolism	Dihydrofolate reductase	(2,41)
InhA	Mycolic acid biosynthesis	NADH-dependent enoyl-[acyl-carrier-protein] reductase	(36,37)
acpM	Fatty acid biosynthesis and meromycolate extension.	Meromycolate extension acyl carrier protein	(21,33)
kasA	Fatty acid biosynthesis and meromycolate extension.	3-oxoacyl-[acyl-carrier protein] synthase	(4,7)
fas	Lipid metabolism	Probable fatty acid synthase	(34)
plsB	Lipid metabolism	Possible acyltransferase	
fabD	Lipid metabolism	Possible malonyl coA-acyl carrier protein transacylase	(18)
fadE	Mycolic acid synthesis	Probable acyl-coA dehydrogenase	(15,29)
accd6	Fatty acid biosynthesis	Acetyl/propionyl-coA carboxylase	(14,29)
Pks13	Synthesis of a polyketide molecule	Probable polyketide beta-ketoacyl synthase	(14)
fbpC	Lipid metabolism	Secreted antigen 85	(39)
Drug transport (Efflux pump) genes			
efpA	Export of drugs	Possible integral membrane efflux protein	(9,12)
iniA	Drug transport	INH inducible protein	(8)
iniB	Drug transport	INH inducible protein	(1)
Mmpl7	Export of antibiotic	Transmembrane transport protein	(11,27,28)
pstB	Active import of inorganic phosphate and export of drugs	Phosphate-transport ATP-binding protein	(32)
Rv1258c	Export of drugs	Conserved membrane protein	(10)
Rv1747	Transport of drug across the membrane.	conserved transmembrane ATP-binding protein	(26)
emrB	Export of drugs	Possible multidrug resistance integral membrane efflux protein	(23)
Rv1819c	Export of drugs	Probable drug-transport transmembrane ATP-binding protein	(5)

4.3 Gene expression analysis in response to INH

QRT-PCR analysis was done to determine the expression levels of the genes listed in table 4.2 following exposure to 0.1 µg/ml INH for 24hrs in an INH susceptible (K636) and INH mono-resistant (R55) *M.tuberculosis* strain with identical Beijing strain genotype according to spoligotyping and IS*6110* DNA fingerprinting.

4.3.1 INH susceptible Beijing strain (K636) MIC (0.1µg/ml)

QRT-PCR analysis of the INH susceptible Beijing strain showed no significant change in expression of 19 genes, whereas 5 genes (*acpM*, *fabD*, *accD6*, *fbpC* and *iniB*) were significantly regulated in response to 0.1µg/ml INH. Among the 5 up-regulated genes, four genes (*acpM*, *fabD*, *accD6* and *fbpC*) were part of the MAP and one (*iniB*) gene was involved in drug transport. Additionally increased fold change was observed in genes that form part of the MAP (Table 4.3).

4.3.2 INH mono-resistant Beijing strain (R55) MIC (4µg/ml)

Analysis of the INH mono-resistance isolate showed significant up-regulation in 8 of the genes (*ahpC*, *acpM*, *fabD*, *accD6*, *efpA*, *iniA*, *iniB*, and *Mmpl7*) and a significant down-regulation in 2 genes (*ndh* and *fbpC*) in response to 0.1 µg/ml INH. Four of the up-regulated genes (*ahpC*, *acpM*, *fabD*, and *accD6*) and 2 of the down-regulated genes (*ndh* and *fpbC*) formed part of the mycolic acid pathway, while 4 of the up-regulated genes (*efpA*, *iniA*, *iniB* and *mmpl7*) were involved in drug transport. Furthermore significant increase in genes expression was observed in genes involved in drug transport (Table 4.3).

Table 4.3: Differential expression profile of genes involved in the mycolic acid pathway and involved in drug transport after exposure to INH.

	INH susceptible (K636)	**INH mono-resistant (R55)**
Genes	***Fold change***	***Fold change***
Mycolic acid pathway genes		
katG	1.1	2.6
furA	1.6	3.9
ahpC	2.5	9.8*
ndh	1.1	-10.6#
InhA	1.0	2.2
acpM	26.9*	6.5*
kasA	1.7	2.1
fas	1.7	2.1
plsB	1.8	2.2
fabD	7.5*	9.7*
fadE	-1.1	2.3
accD6	14.8*	6*
Pks13	1.6	3.2
fbpC	12.5*	-8#
Drug transport genes		
efpA	2.9	15.8*
iniA	2.5	11.3*
iniB	5*	26*
Mmpl7	1.2	5.1*
pstB	2.8	2.1
Rv1258c	2.9	2.7

Legend to table 4.3: Fold change after 24hrs exposure to 0.1µg/ml INH where > 5 fold change was considered significant, # significant down-regulation, *significant up-regulation

Figure 4.4 and 4.5, shows a summarized QRT-PCR analysis results of significantly regulated selected candidate genes from the MAP and drug transport after 24hrs exposure to 0.1µg/ml INH in INH susceptible and INH mono-resistance with no INH gene associated mutation

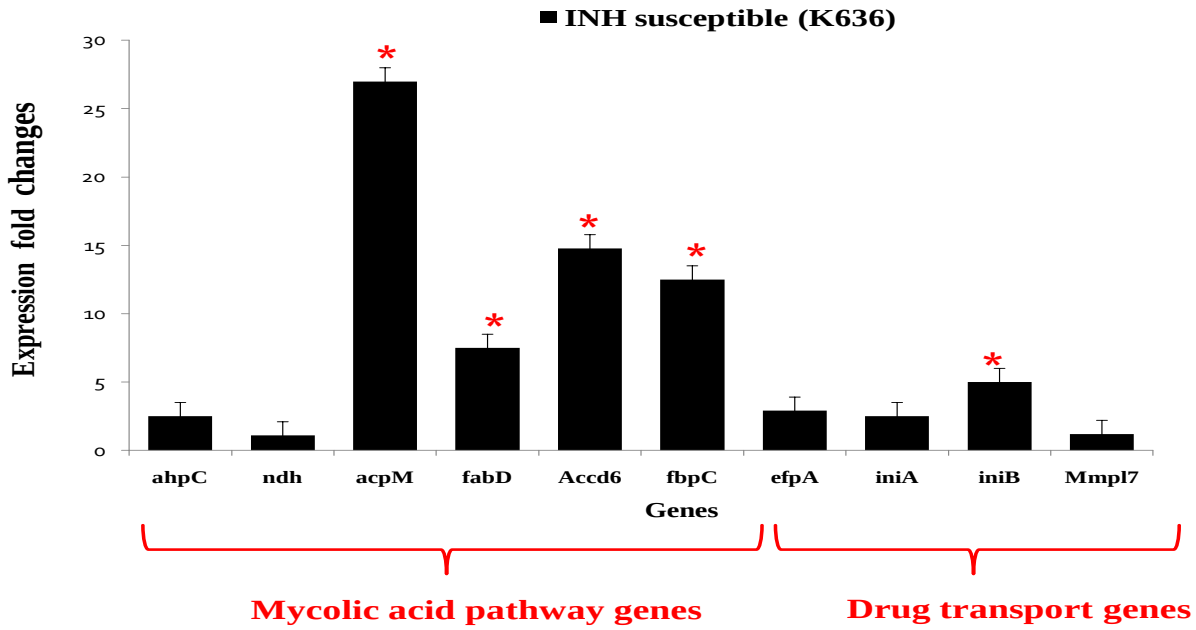

Figure 4.4: Differential gene expression of selected candidate genes after 24hrs INH exposure

Legend to figure 4.4: mRNA expression levels of selected genes in the mycolic acid pathway and drug transport genes in an INH susceptible Beijing strain (INH MIC 0.1µg/ml) after exposure to 0.1µg/ml INH. *** denotes > 5 fold change = significantly differentially expressed genes**

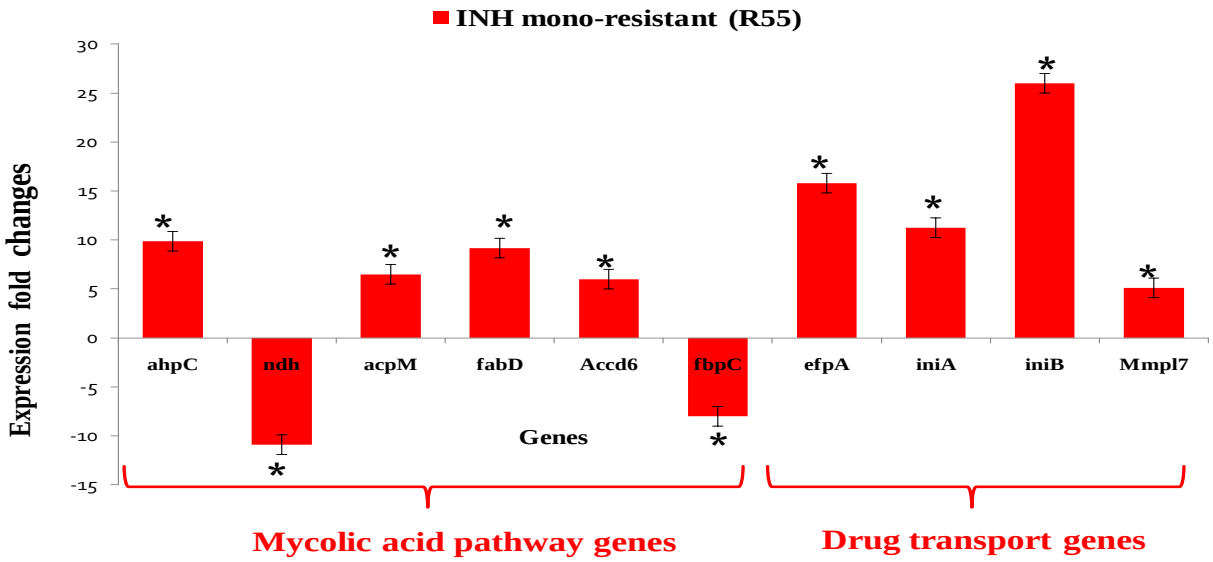

Figure 4.5 Differential gene expressions of selected candidate genes after 24hrs INH exposure

Legend to figure 4.5: mRNA expression levels of selected genes in the mycolic acid pathway and drug transport genes in INH mono resistant Beijing strain (INH MIC 4µg/ml) after exposure to 0.1µg/ml INH. *** denotes > 5 fold change = significantly differentially expressed genes**

Discussion

In this study we selected candidate genes from the MAP, selection of these genes allowed investigation of other genes within the pathway that might be involved in resistance of INH. The MAP genes selected fell into three categories the FAS-I and FAS-II complexes and the final condensation step, without which mycolic acids needed for the survival of the bacterium cannot be synthesized. Additionally, drug transport genes which have been shown to respond to INH treatment were also selected to allow analysis of transport genes as an additional protective mechanism against INH toxicity (8,13,19,31,42).

Exposure of the susceptible strain to INH (0.1µg/ml) resulted in a significant up-regulation in the expression of five genes (*acpM*, *fabD*, *accD6*, *fbpC* and *iniB*), three of the genes belong to the operon-like cluster (*accD6*, *fabD*, *kasA*, *kasB* and *acpM*) encoded by *kas* are involved in extension of meromycolates from FAS-I complex (24,29). It has been shown that inhibition of NADH-dependent enoyl-acyl-carrier-protein (InhA) by INH causes accumulation of mycolates and subsequent up-regulation in the expression of these genes (42). The other expressed gene (*fbpC*) has been implicated in export of proteins that catalysis maturation of mycolic acid in cell wall (35,43) and *iniB* which is a INH inducible genes (1).

QRT-PCR analysis of the selected candidate genes demonstrated a significant change in expression of genes involved in drug transport in INH mono-resistance strain when exposed to INH (0.1µg/ml). This indicates the bacterium intracellular regulation of INH which might be vital for the survival of the bacterium. Additionally a significant down regulation of *ndh* gene was observed indicating inefficient formation of the NAD^+ substrate required for the complex formation with activated INH which results in shielding of InhA from the INH-NAD complex inhibition.

Collectively these QRT-PCR results show that there are differences in genes expressed within the MAP and drug transport genes between the two Beijing isolates after treatment with low INH concentration. For instance, exposure to INH seems to increase expression of MAP genes in the susceptible isolate when compared to the resistant isolate, whereas the transport genes are significantly regulated in the resistance isolates. Furthermore it appears that there is a direct relation in the balance of NAD^+ substrate required for complex formation with INH and activation of transport genes in the resistance isolate, which might be an additional protective mechanism for the bacterium (Figure 4.4 and 4.5). Based on these observations the following models were proposed for the effect of INH in INH susceptible and INH mono-resistant Beijing strains, respectively.

Killing mode of INH in the INH susceptible Beijing isolate

INH enters the cell by passive diffusion where it becomes activated by catalase peroxidase encoded by the *katG* gene (3,17). During the INH activation process reactive oxygen species are formed and the catalase peroxidase on its own is not sufficient to detoxify this reactive oxygen species (40). This imbalance is sensed by the organism resulting in an increase in the synthesis of AhpC, thereby resulting in an increased expression of the *ahpC* gene which assists with detoxification of reactive oxygen species from the cell (35).

The activated INH forms a complex with NAD which targets the inhA protein, thus blocking the FAS-II complex resulting in the accumulation of fatty acid (36). The imbalance in mycolic acid biosynthesis is in turn is sensed by these (acpM-kasA, fabD and accD6) proteins followed by the increase expression of their respective genes (29,42). Furthermore up-regulation of *fbpC* gene due to INH induction is indicative of inhibition of mycolic acid synthesis. Gene expression might be as result of a feedback mechanism that senses the depletion of mature mycolates resulting in the death of the bacterium (42) (Figure 4.6).

Killing mode of INH in the INH susceptible Beijing isolate

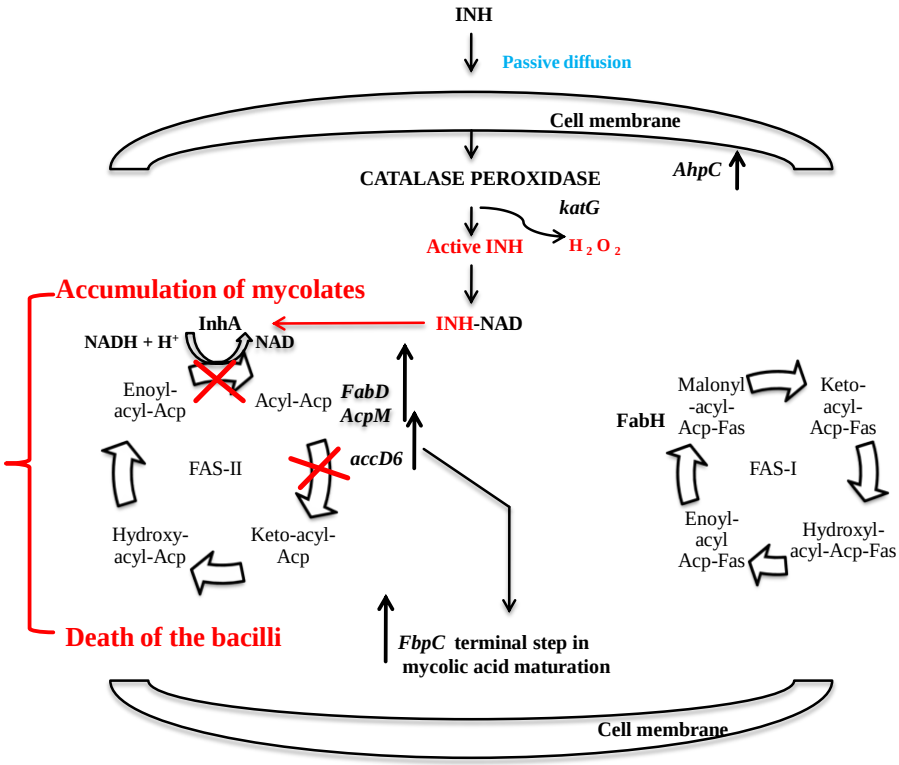

Figure 4.6: Killing mode of sub-lethal concentration of INH in the susceptible Beijing isolate, INH MIC (0.1µg/ml)

Legend to 4.6: ↑ Denotes up-regulation of genes, ✕ denotes reaction inhibition

Mode of INH action in INH mono-resistant Beijing isolate with no associated gene mutations

In the resistant strain, INH enters the cell by passive diffusion where it becomes activated by catalase peroxidase encoded by the *katG* gene (3,17). Similarly to the sensitive strain activation

of INH forms reactive oxygen species and the catalase peroxidase on its own is not sufficient to detoxify this reactive species (40). This imbalance is sensed by the organism resulting in an increase in the synthesis of AhpC, thereby resulting in an increased expression of the *ahpC* gene which assists with detoxification of reactive oxygen species from the cell (42).

Significant down-regulation of *ndh* gene which encodes NADH dehydrogenase was also observed. During the INH activation process NADH dehydrogenase catalysis the conversion of NADH to NAD^+ required for complex formation with the active INH (38). The repressed expression of this gene implies inefficient NAD^+ substrate formation which causes imbalance in intracellular NADH/NAD^+ ratio (38). As a result of this intracellular NADH level increases and acts as a competitive inhibitor to InhA binding, thereby protecting InhA against inhibition by the INH-NAD complex (6,38). At the same time the activated INH results in expression of the drug transporting genes (*efpA*, *iniA*, *iniB* and *Mmpl7*) which causes pumping out of the active INH from the cell, therefore preventing inhibition of the InhA from the INH-NAD complex (Figure 4.7).

INH action in INH mono-resistant Beijing isolate with no INH gene associated mutations

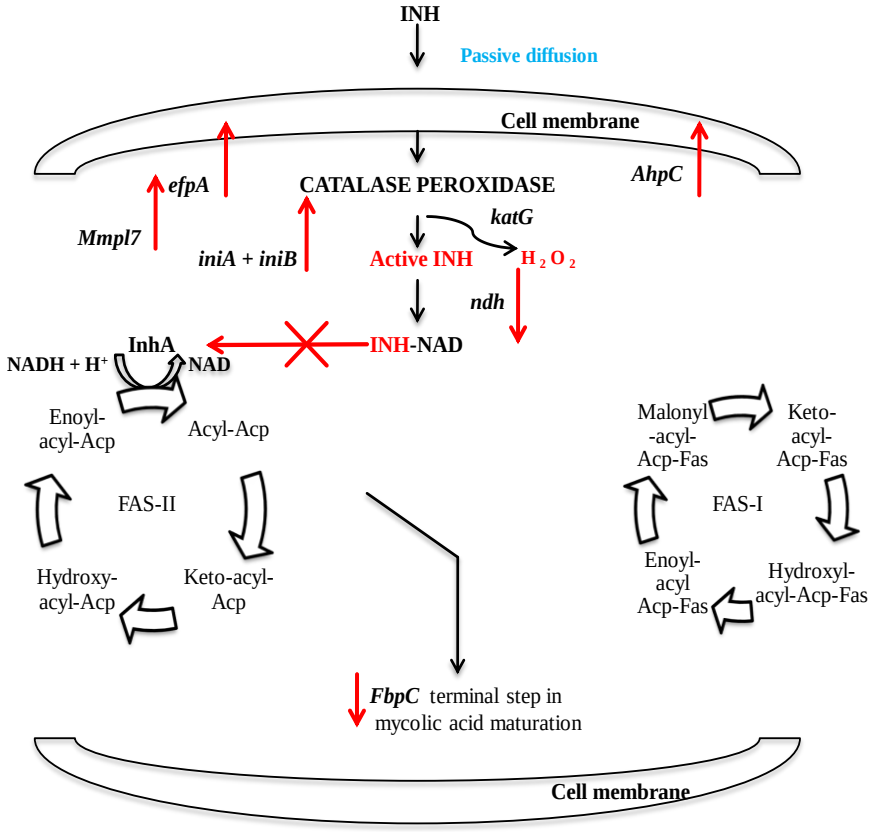

Figure 4.7: Mode of INH action in INH mono-resistant Beijing isolate with no INH gene associated mutations, INH MIC (4µg/ml)

Legend to 4.7: ↑ Denotes up-regulation of genes, ↓ denotes down-regulation of genes, ✕ denotes inhibition of reaction

Figure 4.8, indicates the difference in gene expression observed in the two models. The arrows denote expression of genes for the respective isolates. The gene profile of the susceptible isolate signifies killing mechanism, whereas the mono-resistant isolate presents resistant mechanism.

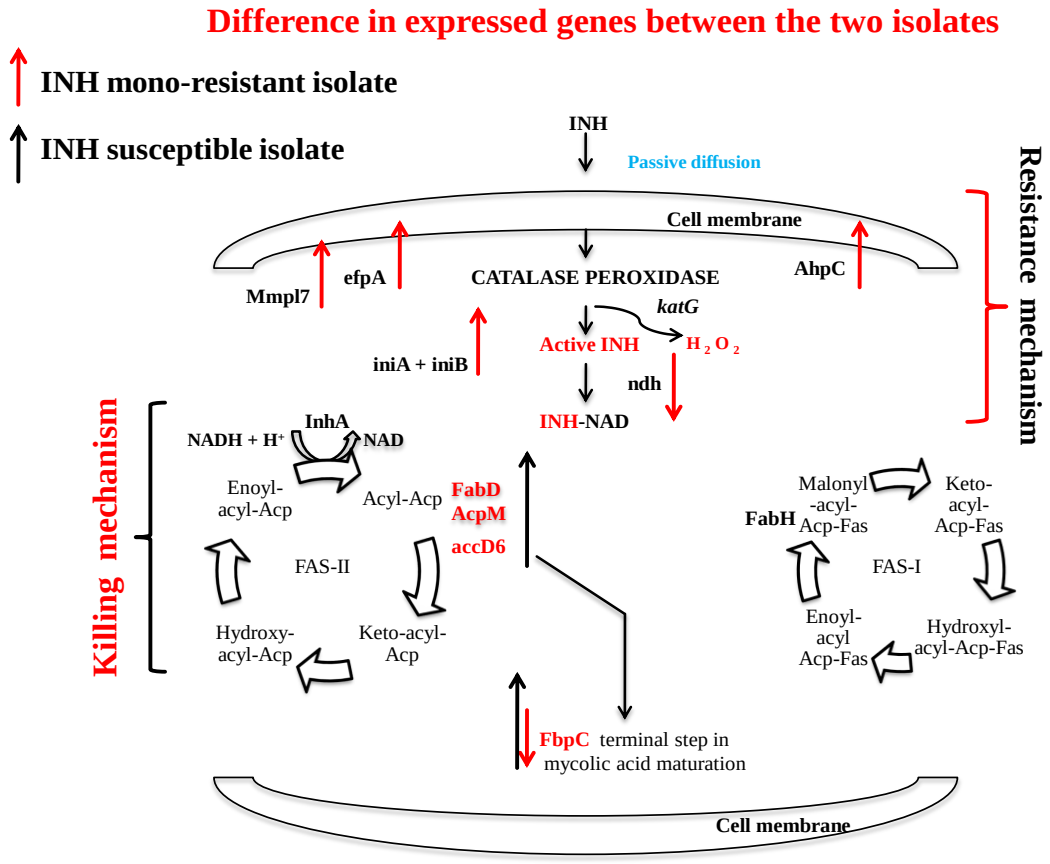

Figure 4.8: Difference observed in gene expression between the two isolates

Legend to 4.8: Genes expressed in INH mono-resistant isolate ↑, Genes expressed in INH susceptible isolate ↑

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Chapter 5

CONCLUSION

Previous microarray studies done on H37Rv and other resistant clinical isolates have reported regulation of various genes after exposure to different concentrations of INH (1-3,5,6). Number of these genes were revealed to encode the FAS-II complex and others to mediated the toxic affect of INH (2-4,6). This study correlates with what has been previously reported in literature, except for the difference in the expression pattern observed in *fbpC* gene between the two isolates and the significant down-regulation of *ndh* gene as a result of INH exposure. In addition this study has shown that the transcriptomics analysis of INH susceptible and INH mono-resistant Beijing isolates revealed various differentially expressed genes in response to low concentrations of INH in both the MAP and drug transport.

The response to INH suggests that expression profiles presented provides characteristic that is specific for the cellular process that is affected by INH and gene expression profile observed in the susceptible isolate is indicative of the inhibition of FAS-II complex indicating death of the bacterium. The proposed model whereby the bacterium compensate for INH toxicity indicate that clinical *Mycobacterium tuberculosis* isolates resistant to INH which do not have genomic mutations might use efflux related mechanism in combination with imbalance in intracellular $NADH/NAD^+$ ratio to prevent inhibition of InhA by INH-NAD complex, thereby allowing the bacterium to acquire additional resistance. However we do acknowledge that our cohort had limitations in that there might be genes from other pathways that directly or indirectly play a role

in INH resistance. Therefore further studies are necessary to test the proposed model which might give a novel insight into the mechanism whereby *Mycobacterium tuberculosis* is capable of evading INH toxicity.

Future studies needed to test our proposed model:

1. Whole genome sequencing analysis of the two isolates in order to understand the complete mechanism of INH in clinical isolates that do not present mutations in the five gene associated with INH

2. RNA sequencing analysis which will enable us to generate an unprecedented global view of the transcriptome between the two strains thus helping us to understand the transcriptome dynamics under INH induction of the selected strains.

3. Utilizing Proteomics to investigate the selected isolate which might reflect the functional importance of a cell in response to INH.

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ADDENDA

Technical issues encounted during the study

1. **Culture growth contamination**

Solution

- All bacterial inoculation was performed in the bio-safety level 3 labs and sterile tissue culture flasks were used.
- Media used was autoclaved and filtered before use
- Culture flasks were wiped with incidin on the outside before storage
- OD measurements were taken once a week and only filtered tips were used

2. **Difficulty in RNA extraction** (DNA contamination, Degraded RNA, Low RNA quantity)

Solution

- Wearing gloves all the time and using RNase wipe to clean all surface
- Using separate stock of chemicals for RNA work
- Filtered RNase free tips and pre packed RNase free materials were used
- Standard PCR was performed using isolated RNA as template to insure that there was no DNA contamination
- Although is virtually impossible to completely eliminate genomic DNA a Minus RT control was also used as an additional control

3. **poor primer design** (primer dimers)

Solution

- Primer design software was used for primers design which included adjustable parameters
- This was followed up by assay validation where the primer pairs were tested in all combination with probe and known template (cDNA)
- Primers that gave the lowest CT and the highest ΔRn were used

- Amplicons were identified by melting curves